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# Taxonomy of Lactobacilli and Bifidobacteria

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## Abstract

Genera *Lactobacillus* and *Bifidobacterium* include a large number of species and strains exhibiting important properties in an applied context, especially in the area of food and probiotics. An updated list of species belonging to those two genera, their phylogenetic relationships and other relevant taxonomic information are reviewed in this paper.

The conventional nature of taxonomy is explained and some basic concepts and terms will be presented for readers not familiar with this important and fast-evolving area, which importance is often underestimated. The analysis of biodiversity and its cataloguing, i.e. taxonomy, constitute the basis for applications and scientific communication: reliable identification and correct naming of bacterial strains are not only primary aims of taxonomic studies, but also fundamental elements in an applied context, for the tracking of probiotic strains and a non fraudulent labelling of fermented milks and pharmaceutical products containing probiotic microorganisms. A number of resources freely available have been listed and their use is suggested for people concerned with different aspects of taxonomy.

Some perspectives in taxonomy have been outlined, in particular considering the role of culture independent analyses to reveal the still unknown and uncultured microorganisms. Finally, the impact of the availability of whole-genome sequences in taxonomy is briefly explained: they have already begun to give insights on bacterial evolution, which will surely have implications on taxonomy, even if the analysis of data for lactic acid bacteria is still limited to few species.

## Introduction

Species of the genera *Lactobacillus* and *Bifidobacterium* are some of the most important taxa involved in food microbiology and human nutrition, due to their role in food and feed production and preservation, and also to the probiotic properties exhibited by some strains. These traits are of increasing importance and receive attention from the consumer and market.

The genus *Lactobacillus* belongs to the Lactic Acid Bacteria (LAB), a definition which groups Gram-positive, catalase-negative bacterial species able to produce lactic acid as main end-product of the fermentation

of carbohydrates. The genus *Bifidobacterium*, even if traditionally listed among LAB, is only poorly phylogenetically related to genuine LAB and its species use a metabolic pathway for the degradation of hexoses different from those described for 'genuine' LAB.

The interest in **what** lactobacilli and bifidobacteria are able to do must consider the investigation of **who** they are.

Before reviewing the taxonomy of those two genera, some basic terms and concepts and preliminary considerations concerning bacterial systematics need to be introduced: they are required for readers who are not familiar with taxonomy to gain a deep understanding of the difficulties in obtaining a clear taxonomic scheme for the bacteria under analysis.

## Basic terms and concepts in bacterial taxonomy

Taxonomy or systematics may be defined as the process of cataloguing biodiversity, as it is the scientific study of the diversity of organisms with the ultimate goal of characterizing and arranging them in an orderly manner (Schleifer and Ludwig, 1994).

Classification, identification and nomenclature are the three separate but related subdisciplines of taxonomy. *Classification* is the process of clustering organisms into taxonomic groups (*taxa*) on the basis of similarities or relationships. *Nomenclature* is the assignment of names to the taxonomic groups according to international rules. Finally, *identification* is the process of determining the belonging of a new isolate to one of the established and named taxa (Staley and Krieg, 1989).

Bacterial taxonomy is an area of growing interest: it has implications for many basic scientific and applied fields and therefore, in some way, it underpins all biological research (Tautz *et al.*, 2003). It is considered to have a 'philosophical' root, derived from human desire to recognize and understand the world, which requires a logical ordering of items (Rosselló-Mora, 2005). Moreover it has a strong practical motivation (Kandler, 1984): *classification* schemes, if reliable, could be predictive and allow quick characterization of new isolates based on similarity with known taxa; *identification* procedures allow confirmation of the identity of strains used, for instance, in patented industrial processes; finally, correct *nomenclature* allows not only scientific communication but also unequivocal labelling of products containing microorganisms, providing customer and producer satisfaction.

Taxa in the classification system are arranged in a hierarchical way. At present, two prokaryotic domains are recognized: Archaea and Bacteria. Domains are divided into phyla and the levels below the phylum are classes, orders (or subdivisions, depending on the group), families, genera and species. Different taxonomic levels are characterized by different suffixes in the taxon names.

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As microorganisms have too simple a structure and too few informative characters compared with higher organisms (e.g. morphology), progress in bacterial taxonomy has always been dependent on advances in technology: modern bacterial taxonomy is built mainly on *molecular* data. These data have been made available with the discovery of DNA as the depositary material of genetic information and the improvement of techniques suitable to deal with the smallest components of cells. Different methods of analysis have different resolution power, therefore the complete investigation of the identity of a microorganism could be carried out only through the comparison of results of a large number of techniques: this is the so-called *polyphasic* approach to bacterial systematics, i.e. the more data you have and compare, the more complete and accurate the identification (Colwell, 1970; Vandamme *et al.*, 1996).

The basic unit of the classification scheme is the *species*. The species concept for prokaryotes is a debated topic and its evolution has been recently reviewed (Rosselló-Mora and Amann, 2001; Gevers *et al.*, 2005) and discussed (see, for instance, Rosselló-Mora, 2003). To date, the most useful and accepted definition for 'species' is the so-called phylo-phenetic concept: a species is considered a monophyletic and genomically coherent cluster of individual organisms that shows a high degree of overall similarity with respect to many independent characteristics, and is diagnosable by a discriminative phenotypic property (Rosselló-Mora and Amann, 2001). A species may be divided into two or more subspecies based on minor but consistent phenotypic variation or on genetically determined clusters of strains within the species (Rosselló-Mora and Amann, 2001); the subspecies is therefore the lowest taxonomic rank with any standing in nomenclature. Key points of species definition are (i) the phylogenetic aspect, i.e. related organisms have a common ancestor, and (ii) the phenetic one, i.e. the overall similarity: the more two taxa are close relatives, the more they should 'resemble' each other. An accepted measure of this resemblance is the similarity of total DNA: two individuals are considered to belong to the same species if they share a DNA–DNA relatedness of 70% or higher (relative binding ratio assay) and/or the hybrid DNA duplex they form in a DNA reassociation assay has a difference in the melting temperature ( $\Delta T_m$ ) equal to 5°C or lower. This threshold has been chosen as it was found to have a good correlation with other data (e.g. phenotypic, chemotaxonomic). In taxonomic practice, DNA–DNA similarity assays have become the gold standard technique for the delineation of bacterial species, but the development of a multilocus sequencing approach has been proposed, which potentialities have not been widely explored yet (Stackebrandt *et al.*, 2002; Gevers *et al.*, 2005).

The definition and naming of *species* is necessary for practical reasons, nevertheless, one should remember that microbiologists work with *strains*, as the strain is the microbial individual. It is possible to assign properties to species when a number of different strains are studied and are homogeneous and, most of all, when the type strain is included in the analysis, but when tests are

performed and properties attributed, they are, first of all, characteristics of the strain under study.

The phylogenetic approach has revolutionized bacterial systematics. Relatedness among organisms is estimated through the comparison of molecular sequences, mainly 16S rRNA encoding genes (Woese, 1987). It is based on several major assumption: (i) rRNA genes are highly conserved because of the fundamental role of ribosomes in protein biosynthesis that was developed in the early stages of the evolution of organisms; (ii) horizontal gene transfer phenomena among organisms have not involved those genes and (iii) the amount of similarity of sequences between different individuals is representative of the variation of their genomes.

Those assumption are not always true or, at least, some doubts have been raised and experimental evidences have been obtained that contradict them. Nevertheless, in general, the 16S rRNA gene analysis has permitted an evolutionary scenario for bacteria to be derived that has been confirmed by the analysis of several other molecules, e.g. proteins such as RecA (Lloyd and Sharp, 1993; Eisen, 1995), elongation factor Tu or ATPase (Ludwig *et al.*, 1993). Obviously, alternative markers should have the same characteristics attributed to ribosomal genes listed above.

The relationship between genomic and 16S rRNA gene sequence divergence has been analysed and an empirical non-linear correlation have been found between total DNA similarity data and percentages of sequence identity (Keswani and Withman, 2001; Rosselló-Mora and Amann, 2001). It could then be affirmed that, in general, if two organisms share a 16S rRNA gene sequence identity lower than 97%, they are poorly related at the genomic level and therefore they belong to different species. If two organisms share identity values higher than 97%, even identical sequences, they have to be considered closely related and only total DNA–DNA hybridization data and/or analysis of other more discriminative gene sequences are decisive for the identification at the species level.

Phylogenetic analysis is largely preferred over DNA hybridization tests because the latter are time-consuming, and are complex and expensive. In contrast, identification through sequence analysis is based on specific DNA amplification, sequencing reaction and comparison with public databases, which are very fast, more reproducible and much less expensive procedures (Gevers *et al.*, 2005).

Once the phylogenetic placement of an individual has been elucidated, its relationships to its closest relatives has to be elucidated through other data (phenotypic, metabolic, chemotaxonomic) in the already mentioned polyphasic way. If it constitutes a new species, it has also to be named according to international rules, defined in the International Code of Nomenclature of Bacteria, or Bacteriological Code (Lapage *et al.*, 1992), and validly published. Valid publication includes the indication of the *type* strain, which is the strain to be considered permanently linked to the name of the species. The type strain should therefore be included in all the comparative studies among different species.

Several other resources concerning the taxonomy of bacteria and of *Lactobacillus* and *Bifidobacterium* genera in particular are reported at the end of the paper.

After this very concise summary of approaches and basic terms and concepts of bacterial systematics, it should be clear that taxonomy of an organism is neither a simple nor a quickly resolved task and, most of all, that it is based on a series of conventional thresholds (DNA–DNA hybridization values, sequence similarity values), reference points (type strains) and concepts (foremost, the species concept). Moreover the fundamental pre-requisite for taxonomy is the isolation of the microorganisms in pure culture. Uncultured biodiversity exists and it constitutes the great majority of the existing biodiversity.

Finally, it is important to say that the taxonomic picture we are presenting here, and in general the taxonomic scenario for microorganisms, is evolving and it is destined to change in time. Biodiversity exists but classification schemes are conventional, based on a species concept and threshold values for species delimitation which are artificial even if pragmatic and useful. Thus progress in science, new evidences and new data will surely contribute to change the data we are presenting here.

### The genus *Lactobacillus*

Lactobacilli are Gram-positive, non-spore-forming microorganisms. Considering cellular shape, they can occur as rods or coccobacilli. They are fermentative, microaerophilic and chemo-organotrophic, requiring rich media to grow. They are catalase negative, even if pseudocatalase activity can sometimes be present in some strains. Considering DNA base composition of the genome, they usually show a GC content of lower than 54 mol%.

They are almost ubiquitous: they are found in environments where carbohydrates are available, such as food (dairy products, fermented meat, sour doughs, vegetables, fruits, beverages), respiratory, GI and genital tracts of humans and animals, and in sewage and plant material.

According to *Taxonomic Outline of the Prokaryotes* (Release 5.0, Garrity *et al.*, 2004), the genus *Lactobacillus* belongs to the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales*, family *Lactobacillaceae* and its closest relatives, being grouped within the same family, are the genera *Paralactobacillus* and *Pediococcus*.

The phylogenetically closest Family appears to be the *Leuconostocaceae* family, which includes genera *Leuconostoc*, *Oenococcus* and *Weissella* (Hammes and Hertel, 2003).

At the time of writing (February 2007), the genus *Lactobacillus* includes 106 validly described species, and it is, therefore, the most numerous genus of the order

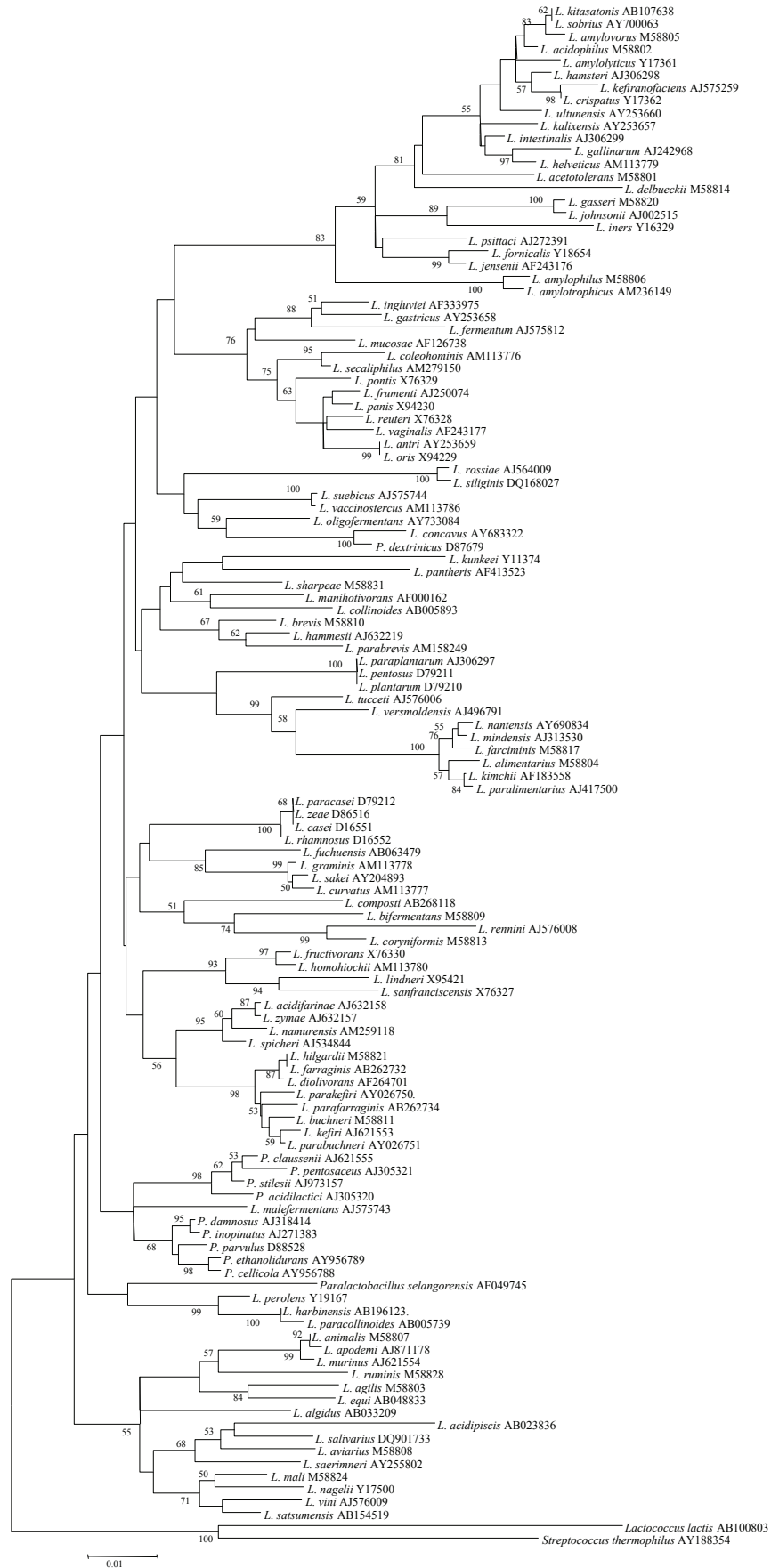
*Lactobacillales*. Moreover, 1 species has been described but it has not been validated yet (*Lactobacillus tucseti*, Chenoll *et al.*, 2006), and few other species description are about to be published (*Lactobacillus composti*, *Lactobacillus farraginis*, and *Lactobacillus parafarraginis*, Endo and Okada, 2007a, 2007b; *Lactobacillus secaliphilus*, Ehrmann *et al.*, 2007, <http://ijls.sgmjournals.org/misc/pip.shtml>).

Seven species in the genus *Lactobacillus* comprise 2 subspecies or more: *Lactobacillus aviarius* (*L. aviarius* subsp. *aviarius* and *L. aviarius* subsp. *araffinosus*), *Lactobacillus coryniformis* (*L. coryniformis* subsp. *coryniformis* and *L. coryniformis* subsp. *torquens*), *Lactobacillus delbrueckii* (*L. delbrueckii* subsp. *delbrueckii*, *L. delbrueckii* subsp. *bulgaricus*, *L. delbrueckii* subsp. *indicus*, and *L. delbrueckii* subsp. *lactis*), *Lactobacillus kefiranofaciens* (*L. kefiranofaciens* subsp. *kefiranofaciens* and *L. kefiranofaciens* subsp. *kefirgranum*), *Lactobacillus paracasei* (*L. paracasei* subsp. *paracasei* and *L. paracasei* subsp. *tolerans*), *Lactobacillus plantarum* (*L. plantarum* subsp. *plantarum* and *L. plantarum* subsp. *argenteratensis*), and *Lactobacillus sakei* (*L. sakei* subsp. *sakei* and *L. sakei* subsp. *carneus*), while the insubstantial separation in two subspecies for *L. salivarius* has been recently demonstrated and species description emended (Li *et al.*, 2006). Genera *Paralactobacillus* and *Pediococcus* are constituted by 1 and 10 species, respectively and no subspecies is known for them.

The phylogenetic structure of the Family *Lactobacillaceae* is reported in Fig. 1, taking *Lactococcus lactis* and *Streptococcus thermophilus* as outgroups, i.e. the reference sequences. The species *Lactobacillus catenaformis* and *Lactobacillus vitulinus* have not been included: it is known that they are only poorly related to the genus *Lactobacillus* (Pot *et al.*, 1994; Hammes and Vogel, 1995; Hammes and Hertel, 2003) and their closest relatives seem to be *Catenibacterium* (Kageyama and Benno, 2000a) and *Coprobacillus* (Kageyama and Benno, 2000b). *Lactobacillus rogosae* could not be included in the analysis because, to date, no strain is available that corresponds to the original description of the species; therefore the species can be considered invalid (Felis *et al.*, 2004). The first phylogenetic analysis of lactobacilli was performed in 1991 by Collins and co-workers, on the smaller number of species known at that time: they suggested to subdivide the genus *Lactobacillus* into three groups: the *Lactobacillus delbrueckii* group, the *Lactobacillus casei-Pediococcus* group and the *Leuconostoc* group, which also contained some lactobacilli.

Schleifer and Ludwig (1995) confirmed these findings and *L. delbrueckii* group was given the name of *L. acidophilus* group; even if *L. delbrueckii* is the type species of the genus *Lactobacillus*, it was not as representative of

Fig. 1 (opposite) Phylogenetic tree based on 16S rRNA gene sequence analysis depicting the phylogenetic relationships among species of the genera *Lactobacillus*, *Pediococcus* and *Paralactobacillus*. In this representation, the phylogenetic distance between taxa is given by the sum of horizontal branch lengths. Bar indicates number of nucleotide substitution per site. Different trees have been obtained as follows: sequences have been aligned with MUSCLE software (<http://www.ebi.ac.uk/muscle/>) or ClustalW (<http://www.ebi.ac.uk/clustalw/index.html>), and trees have been inferred with MEGA software version 3.1 (<http://www.megasoftware.net/>) with different option for distance matrix calculation (Kimura, Tamura 3 parameters) and tree reconstruction (neighbour joining and minimum evolution). Results obtained with different options were very similar, and, in the figure, the tree obtained after alignment with MUSCLE, and tree inference with Tamura 3 parameters and minimum evolution. Statistical support of the clades has been estimated with bootstrap (1000 replicates), which percentages are reported at the nodes (only values above 50% are reported). Usually only values above 70–80% are considered to indicate high support, in this case we considered values above 50 to avoid dispersion of species.



its phylogenetic group as *L. acidophilus*. Moreover, these authors noted that the *L. casei*-*Pediococcus* group could be split into a further four subclusters. The description of a large number of species in recent years and the following phylogenetic re-examination of the genus have made splitting these groups into smaller groups more feasible. This strategy of groupings have been adopted from Hammes and Hertel (2003), and Dellaglio and Felis (2005), have been updated here, and are summarized in Table 1.

The main discrepancy in the taxonomy of the genus *Lactobacillus* is the non-correlation between phylogenetic placement and metabolic properties. The historical subdivisions of the genus *Lactobacillus* based on the type of fermentation have been excellently reviewed by Pot *et al.* (1994), who have underlined how terms such as 'homofermentative', 'heterofermentative', 'obligately homofermentative', 'facultatively heterofermentative' and 'obligately heterofermentative' have been given different meanings by different authors and may be misleading. The accepted 'modern' definition is that given by Hammes and Vogel (1995): **obligately homofermentative** lactobacilli are able to ferment hexoses almost exclusively to lactic acid by the Embden–Meyerhof–Parnas (EMP) pathway while pentoses and gluconate are not fermented as they lack phosphoketolase; **facultatively heterofermentative** lactobacilli degrade hexoses to lactic acid by the EMP pathway and are also able to degrade pentoses and often gluconate as they possess both aldolase and phosphoketolase; finally, **obligately heterofermentative** degrade hexoses by the phosphogluconate pathway producing lactate, ethanol or acetic acid and carbon dioxide; moreover, pentoses are fermented by this pathway.

All valid species of the genus *Lactobacillus* are shown in Table 2, together with metabolic characteristics, phylogenetic grouping, genome GC content and type of the peptidoglycan of the cell wall. Fermentation profiles were not considered as they are not useful for identification purposes and they can easily be found in previous reviews on the taxonomy of lactic acid bacteria (Kandler and Weiss, 1989; Dellaglio *et al.*, 1994; Pot *et al.*, 1994; Hammes and Vogel, 1995) and in papers describing novel species.

Phylogenetic structure of the genus *Lactobacillus* is quite complicated, a representative tree is reported in Fig. 1. The combination of different methods of phylogenetic analysis and different models of phylogenetic inference allowed the recognition of a number of phylogenetic groups. In Table 1, the composition of phylogenetic groups is reported according to different authors. It can be noted that the addition of novel species dramatically changes the phylogenetic structure of the genus even in a short time.

The most noteworthy changes with respect to previous surveys are:

- the association of *L. buchneri* subgroup-a (Dellaglio and Felis, 2005) with a different subgroup (Table 2). This is probably due to the description of *L. farraginis*, *L. parafarraginis*, *L. namurensis*, and *L. parabrevis*: the addition of this novel taxa has determined a

reorganization of the scenario and the emergence of different clades

- the definition of *L. alimentarius*-*L. farciminis* group, probably due to the description of *L. tucseti* and *L. nantensis*
- the reduction of the *L. casei* group described by Hammes and Hertel (2003), to only four species
- the separation of the genus *Pediococcus* into two clades, very often not robustly associated, and the association of *P. dextrinicus* to *L. concavus*.

Nowadays, the fastest way to identify lactobacilli, as well as bifidobacteria and bacteria in general, is the partial or complete sequencing of 16S rRNA gene and the evaluation of patterns of fermentations and other characteristics in relation only to the closest relatives. It must be remembered that the identification based on 16S rRNA gene may be misleading if closely related species are analysed. As a slowly diverging molecule, 16S rRNA is not able to reveal significant differences between recently diverged species such as *L. plantarum*, *L. paraplantarum* and *L. pentosus*, or *L. casei*, *L. rhamnosus* and *L. zeae*. In those cases, 16S rRNA gene sequence analysis can indicate only the belonging to a group, not to a definite species. To obtain a more detailed identification, even at subspecies level, sequencing of protein-encoding genes may be undertaken successfully (e.g. *recA* gene for *L. casei* and *L. plantarum*: Felis *et al.*, 2001, Torriani *et al.*, 2001, Bringel *et al.*, 2005), even if DNA–DNA hybridization is the resolutive technique. Other very interesting taxonomic parameters for the genus *Lactobacillus* are the genome GC content, the type of isomer of lactic acid produced and the composition of peptidoglycan of the cell wall.

The wide range of genome GC content of the described species is a interesting and contradictory feature of the genus *Lactobacillus*: it ranges from 32 to 54 mol%, which is a span about twice as large as that normally accepted for well defined genera (Schleifer and Ludwig, 1995). Genome GC content is thought to be an evolution-linked trait so that sister taxa are usually characterized by similar GC contents (Graur and Li, 1999). This seems not to be the case for lactobacilli; even considering the most well-defined phylogenetic subgroup of the genus, i.e. the *L. delbrueckii*-*L. acidophilus* group, the genome GC content still ranges from 32% to 50% (Table 1).

It should be clear, from the reported data, that the taxonomy of lactobacilli is far from being well established and satisfactory. Besides the reviewed aspects of genus classification, open issues regarding particular species exist, which could have important implications for identification and nomenclature of strains. The most important case is that of the nomenclature of the species *L. casei*. The emended description of *L. casei* (Orla-Jensen 1919) comb. nov. by Hansen and Lessel (1971) designated ATCC 393 as the neotype strain of *L. casei* subsp. *casei*, on the basis of few phenotypic traits. Shortly after its designation, ATCC 393<sup>T</sup> was shown to be unsuitable as the neotype strain of *L. casei* on the basis of DNA/DNA hybridization experiments (Mills and Lessel, 1973; Dellaglio *et al.*, 1975), as it shared very high genome similarity (84%) with *Lactobacillus casei* subsp. *rhamnosus* ATCC 15820, former type strain of '*Lactobacterium zeae*'



**Table 2** Some relevant taxonomic data of the species of the genus *Lactobacillus*: full species names, type of glucose fermentation, phylogenetic grouping according to the present re-analysis, genome GC content, cell wall composition, and lactic acid isomer produced through fermentation. For species not yet published or validated, only the species name is reported

Full species name	Metabolism <sup>a</sup>	Phylogenetic group <sup>b</sup>	GC content	Peptidoglycan	Lactic acid isomer
<i>Lactobacillus acetotolerans</i> Entani <i>et al.</i> 1986	B	delb	35–37	Lys-D-Asp	DL
<i>Lactobacillus acidifarinae</i> Vancanneyt <i>et al.</i> 2005	C	buch	51	nd	DL
<i>Lactobacillus acidipiscis</i> Tanasupawat <i>et al.</i> 2000	B	sal	38–41	Lys-D-Asp	L
<i>Lactobacillus acidophilus</i> (Moro 1900) Hansen and Mocquot 1970	A	delb	34–37	Lys-D-Asp	DL
<i>Lactobacillus agilis</i> Weiss <i>et al.</i> 1982	B	sal	43–44	DAP	L
<i>Lactobacillus algidus</i> Kato <i>et al.</i> 2000	B	sal-ss	36–37	DAP	L
<i>Lactobacillus alimentarius</i> Reuter 1983	B	al-far	36–37	Lys-D-Asp	L-DL
<i>Lactobacillus amyolyticus</i> Bohak <i>et al.</i> 1999	A	delb	39	Lys-D-Asp	DL
<i>Lactobacillus amylophilus</i> Nakamura and Crowell 1981	A	delb	44–46	Lys-D-Asp	L
<i>Lactobacillus amylophilus</i> Naser <i>et al.</i> 2006	A	delb	43.5	nd	L
<i>Lactobacillus amylovorus</i> Nakamura 1981	A	delb	40–41	Lys-D-Asp	DL
<i>Lactobacillus animalis</i> Dent and Williams 1983	A	sal	41–44	Lys-D-Asp	L
<i>Lactobacillus antri</i> Roos <i>et al.</i> , 2005	C	reu	44–45	Lys-D-Asp	DL
<i>Lactobacillus apodemi</i> Osawa <i>et al.</i> 2006	B	sal	38.5	L-Lys-D-Asp	L
<i>Lactobacillus aviarius</i> subsp. <i>araffinosus</i> Fujisawa <i>et al.</i> 1986	A	sal	39–43	Lys-D-Asp	DL (D <15%)
<i>Lactobacillus aviarius</i> subsp. <i>aviarius</i> Fujisawa <i>et al.</i> 1986	A	sal	39–43	Lys-D-Asp	DL
<i>Lactobacillus bifementans</i> Kandler <i>et al.</i> 1983	B	cor	45	Lys-D-Asp	DL
<i>Lactobacillus brevis</i> (Orla-Jensen 1919) Bergey <i>et al.</i> 1934	C	bre	44–47	Lys-D-Asp	DL
<i>Lactobacillus buchneri</i> (Henneberg 1903) Bergey <i>et al.</i> 1923	C	buch	44–46	Lys-D-Asp	DL
<i>Lactobacillus casei</i> (Orla-Jensen 1916) Hansen and Lessel 1971	B	cas	45–47	Lys-D-Asp	L
<i>Lactobacillus catenaformis</i> (Eggerth 1935) Moore and Holdeman 1970	A	see text	31–33	Lys-Ala	D
<i>Lactobacillus coleohominis</i> Nikolaitchouk <i>et al.</i> 2001	B	reu	nd	mDAP	DL
<i>Lactobacillus collinoides</i> Carr and Davies 1972	C	couple3	46	Lys-D-Asp	DL
<i>Lactobacillus compostii</i> Endo and Okada 2007	B	cor	48	no mDAP	DL
<i>Lactobacillus concavus</i> Tong and Dong 2005	A	Pdex	46–47	mDAP	DL (D 5%)
<i>Lactobacillus coryniformis</i> subsp. <i>coryniformis</i> Abo-Elnaga and Kandler 1965	B	cor	45	Lys-D-Asp	DL (L <15%)
<i>Lactobacillus coryniformis</i> subsp. <i>torquens</i> Abo-Elnaga and Kandler 1965	B	cor	45	Lys-D-Asp	D
<i>Lactobacillus crispatus</i> (Brygoo and Aladame 1953) Moore and Holdeman 1970	A	delb	35–38	Lys-D-Asp	DL
<i>Lactobacillus curvatus</i> (Troili-Petersson 1903) Abo-Elnaga and Kandler 1965 emend. Klein <i>et al.</i> 1996	B	sakei	42–44	Lys-D-Asp	DL
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> (Orla-Jensen 1919) Weiss <i>et al.</i> 1984	A	delb	49–51	Lys-D-Asp	D
<i>Lactobacillus delbrueckii</i> subsp. <i>delbrueckii</i> (Leichmann 1896) Beijerinck 1901	A	delb	49–51	Lys-D-Asp	D
<i>Lactobacillus delbrueckii</i> subsp. <i>indicus</i> Dellaglio <i>et al.</i> , 2005	A	delb	nd	nd	D
<i>Lactobacillus delbrueckii</i> subsp. <i>lactis</i> (Orla-Jensen 1919) Weiss <i>et al.</i> 1984	A	delb	49–51	Lys-D-Asp	D
<i>Lactobacillus diolivorans</i> Krooneman <i>et al.</i> 2002	C	buch	nd	nd	nd
<i>Lactobacillus equi</i> Morotomi <i>et al.</i> 2002	A	sal	38–39	nd	DL
<i>Lactobacillus farciminis</i> Reuter 1983	A	al-far	34–36	Lys-D-Asp	L (D <15%)
<i>Lactobacillus farraginis</i> Endo and Okada 2007	B	buch	40–41	no mDAP	DL
<i>Lactobacillus fermentum</i> Beijerinck 1901 emend. Dellaglio <i>et al.</i> 2004	C	reu	52–54	Orn-D-Asp	DL
<i>Lactobacillus formicalis</i> Dicks <i>et al.</i> 2000	B	delb	37	nd	DL
<i>Lactobacillus fructivorans</i> Charlton <i>et al.</i> 1934	C	fru	38–41	Lys-D-Asp	DL
<i>Lactobacillus frumenti</i> Müller <i>et al.</i> 2000	C	reu	43–44	Lys-D-Asp	L

Table 2 continued

Full species name	Metabolism <sup>a</sup>	Phylogenetic group <sup>b</sup>	GC content	Peptidoglycan	Lactic acid isomer
<i>Lactobacillus fuchuensis</i> Sakala <i>et al.</i> 2002	B	sakei	41–42	nd	L (D<40%)
<i>Lactobacillus gallinarum</i> Fujisawa <i>et al.</i> 1992	A	delb	36–37	Lys-D-Asp	DL
<i>Lactobacillus gasserii</i> Lauer and Kandler 1980	A	delb	33–35	Lys-D-Asp	DL
<i>Lactobacillus gastricus</i> Roos <i>et al.</i> , 2005	C	reu	41–42	L-Orn-D-Asp	DL
<i>Lactobacillus graminis</i> Beck <i>et al.</i> 1989	B	sakei	41–43	Lys-D-Asp	DL
<i>Lactobacillus hammesii</i> Valcheva <i>et al.</i> 2005	B	bre	nd	L-Lys-D-Asp	DL
<i>Lactobacillus hamsteri</i> Mitsuoka and Fujisawa 1988	B	delb	33–35	Lys-D-Asp	DL
<i>Lactobacillus harbinensis</i> Miyamoto <i>et al.</i> 2006	B	per	53–54	nd	L
<i>Lactobacillus helveticus</i> (Orla-Jensen 1919) Bergey <i>et al.</i> 1925	A	delb	38–40	Lys-D-Asp	DL
<i>Lactobacillus hilgardii</i> Douglas and Cruess 1936	C	buch	39–41	Lys-D-Asp	DL
<i>Lactobacillus homohiochii</i> Kitahara <i>et al.</i> 1957	B	fru	35–38	Lys-D-Asp	DL
<i>Lactobacillus iners</i> Falsen <i>et al.</i> 1999	A	delb	34–35	Lys-D-Asp	L
<i>Lactobacillus ingluviei</i> Baele <i>et al.</i> 2003	C	reu	49–50	nd	nd
<i>Lactobacillus intestinalis</i> (ex Hemme 1974) Fujisawa <i>et al.</i> 1990	B	delb	33–35	Lys-D-Asp	DL
<i>Lactobacillus jensenii</i> Gasser <i>et al.</i> 1970	B	delb	35–37	Lys-D-Asp	D
<i>Lactobacillus johnsonii</i> Fujisawa <i>et al.</i> 1992	A	delb	33–35	Lys-D-Asp	DL
<i>Lactobacillus kalixensis</i> Roos <i>et al.</i> , 2005	A	delb	35–36	Lys-D-Asp	DL
<i>Lactobacillus kefiranofaciens</i> subsp. <i>kefiranofaciens</i> Fujisawa <i>et al.</i> 1988	A	delb	34–38	nd	DL
<i>Lactobacillus kefiranofaciens</i> subsp. <i>kefirgranum</i> (Takizawa <i>et al.</i> 1994) Vancanneyt <i>et al.</i> 2004	A	delb	34–38	nd	DL
<i>Lactobacillus kefirii</i> Kandler and Kunath 1983	C	buch	41–42	Lys-D-Asp	DL
<i>Lactobacillus kimchii</i> Yoon <i>et al.</i> 2000	B	al-far	35	nd	DL
<i>Lactobacillus kitasatonis</i> Mukai <i>et al.</i> 2003	B	delb	37–40	nd	DL
<i>Lactobacillus kunkeei</i> Edwards <i>et al.</i> 1998	C	ss	nd	Lys-D-Asp	L
<i>Lactobacillus lindneri</i> Back <i>et al.</i> 1997	C	fru	35	Lys-D-Asp	DL
<i>Lactobacillus malefermentans</i> Farrow <i>et al.</i> 1989	C	ss	41–42	Lys-D-Asp	nd
<i>Lactobacillus mali</i> Carr and Davies 1970, emend. Kaneuchi <i>et al.</i> , 1998	A	sal	32–34	DAP	L
<i>Lactobacillus manihotivorans</i> Morlon-Guyot <i>et al.</i> 1998	A	couple3	48–49	nd	L
<i>Lactobacillus mindensis</i> Ehrmann <i>et al.</i> 2003	A	al-far	37–38	Lys-D-Asp	DL
<i>Lactobacillus mucosae</i> Roos <i>et al.</i> 2000	C	reu	46–47	Orn-D-Asp	DL
<i>Lactobacillus murinus</i> Hemme <i>et al.</i> 1982	B	sal	43–44	Lys-D-Asp	L
<i>Lactobacillus nagelii</i> Edwards <i>et al.</i> 2000	A	sal	nd	nd	DL
<i>Lactobacillus namurensis</i> Scheirlinck <i>et al.</i> 2007	C	buch	52	nd	DL
<i>Lactobacillus nantensis</i> Valcheva <i>et al.</i> 2006	B	al-far	38.6	nd	DL
<i>Lactobacillus oligofermentans</i> Koort <i>et al.</i> 2005	C	Pdex	35.3–39.9	nd	DL (D 30%)
<i>Lactobacillus oris</i> Farrow and Collins 1988	C	reu	49–51	Orn-D-Asp	DL
<i>Lactobacillus panis</i> Wiese <i>et al.</i> 1996	C	reu	49–51	Lys-D-Asp	DL
<i>Lactobacillus pantheris</i> Liu and Dong 2002	A	ss	52–53	nd	D
<i>Lactobacillus parabrevis</i> Vancanneyt <i>et al.</i> 2006	C	bre	49	nd	DL
<i>Lactobacillus parabuchneri</i> Farrow <i>et al.</i> 1989	C	buch	44	Lys-D-Asp	nd
<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i> Collins <i>et al.</i> 1989	B	cas	45–47	Lys-D-Asp	L
<i>Lactobacillus paracasei</i> subsp. <i>tolerans</i> Collins <i>et al.</i> 1989	B	cas	45–47	Lys-D-Asp	L
<i>Lactobacillus paracollinoides</i> Suzuki <i>et al.</i> 2004	C	per	44–45	nd	D
<i>Lactobacillus parafarraginis</i> Endo and Okada 2007	B	buch	40	no mDAP	DL (D < 70%)
<i>Lactobacillus parakefirii</i> Takizawa <i>et al.</i> 1994	C	buch	41–42	nd	L
<i>Lactobacillus paralimentarius</i> Cai <i>et al.</i> 1999	B	al-far	37–38	nd	nd

Table 2 continued

Full species name	Metabolism <sup>a</sup>	Phylogenetic group <sup>b</sup>	GC content	Peptidoglycan	Lactic acid isomer
<i>Lactobacillus parapantarum</i> Curk <i>et al.</i> 1996	B	plan	44–45	DAP	DL
<i>Lactobacillus pentosus</i> Zanoni <i>et al.</i> 1987	B	plan	46–47	DAP	DL
<i>Lactobacillus perolens</i> Back <i>et al.</i> 2000	B	per	49–53	Lys-D-Asp	L
<i>Lactobacillus plantarum</i> (Orla-Jensen 1919) Bergey <i>et al.</i> 1923	B	plan	44–46	DAP	DL
<i>Lactobacillus plantarum</i> subsp. <i>argenteratensis</i> Bringel <i>et al.</i> 2005	B	plan	44–46	nd	DL
<i>Lactobacillus pontis</i> Vogel <i>et al.</i> 1994	C	reu	53–56	Orn-D-Asp	DL
<i>Lactobacillus psittaci</i> Lawson <i>et al.</i> 2001	C	delb	nd	nd	nd
<i>Lactobacillus rennini</i> Chenoll <i>et al.</i> 2006	B	cor	nd	L-Lys-D-Asp	DL
<i>Lactobacillus reuteri</i> Kandler <i>et al.</i> 1982	C	reu	40–42	Lys-D-Asp	DL
<i>Lactobacillus rhamnosus</i> (Hansen 1968) Collins <i>et al.</i> 1989	B	cas	45–47	Lys-D-Asp	L
<i>Lactobacillus rogosae</i> Holdeman and Moore 1974	na	na	na	na	na
<i>Lactobacillus rossiae</i> Corsetti <i>et al.</i> 2005	C	couple1	44–45	Lys-Ser-Ala <sub>2</sub>	DL
<i>Lactobacillus ruminis</i> Sharpe <i>et al.</i> 1973	A	sal	44–47	DAP	L
<i>Lactobacillus saerimneri</i> Pedersen and Roos, 2004	A	sal	42–43	DAP	DL
<i>Lactobacillus sakei</i> subsp. <i>carneus</i> Torriani <i>et al.</i> 1996	B	sakei	42–44	nd	DL
<i>Lactobacillus sakei</i> subsp. <i>sakei</i> Katagiri <i>et al.</i> 1934 emend. Klein <i>et al.</i> 1996	B	sakei	42–44	nd	DL
<i>Lactobacillus salivarius</i> Rogosa <i>et al.</i> 1953 emend. Li <i>et al.</i> 2006	A	sal	34–36	Lys-D-Asp	L
<i>Lactobacillus sanfranciscensis</i> Weiss and Schillinger 1984	C	fru	36–38	Lys-Ala	DL
<i>Lactobacillus satsumensis</i> Endo and Okada, 2005	A	sal	39–41	DAP	L
<i>Lactobacillus secaliphilus</i> Ehrmann <i>et al.</i> 2007	B	reu	48	L-Lys-D-Asp	L (D 5%)
<i>Lactobacillus sharpeae</i> Weiss <i>et al.</i> 1982	A	ss	53	DAP	L
<i>Lactobacillus siliginis</i> Aslam <i>et al.</i> 2006	C	couple1	44.5	L-Lys-D-Glu-L-Ala	nd
<i>Lactobacillus sobrius</i> Konstantinov <i>et al.</i> 2006	B	delb	35–36	nd	DL
<i>Lactobacillus spicheri</i> Meroth <i>et al.</i> 2004	B	buch	55	Lys-D-Asp	DL
<i>Lactobacillus suebicus</i> Kleynmans <i>et al.</i> 1989	C	couple2	40–41	DAP	DL
<i>Lactobacillus tucseti</i>	A	al-far	nd	L-Lys-Gly-D-Asp	DL
<i>Lactobacillus ultunensis</i> Roos <i>et al.</i> , 2005	A	delb	35–36	Lys-D-Asp	DL
<i>Lactobacillus vaccinostercus</i> Kozaki and Okada 1983	C	couple2	36–37	DAP	nd
<i>Lactobacillus vaginalis</i> Embley <i>et al.</i> 1989	C	reu	38–41	Orn-D-Asp	nd
<i>Lactobacillus versmoldensis</i> Kröckel <i>et al.</i> 2003	A	al-far	40–41	nd	L
<i>Lactobacillus vini</i> Rodas <i>et al.</i> 2006	B	sal	39.4	L-Lys-D-Asp	DL
<i>Lactobacillus vitulinus</i> Sharpe <i>et al.</i> 1973	A	see text	34–37	mDAP	D
<i>Lactobacillus zaeae</i> Dicks <i>et al.</i> 1996	B	cas	48–49	Lys-D-Asp	L
<i>Lactobacillus zymae</i> Vancanneyt <i>et al.</i> 2005	C	buch	53–54	nd	DL

<sup>a</sup>Type of glucose fermentation, following the conventions of Hammes and Vogel (1995) and Hammes and Hertel (2003), metabolism is indicated with A, B and C capital letters for obligately homofermentative (A), facultatively heterofermentative (B), and obligately heterofermentative (C), respectively.

<sup>b</sup>Abbreviations defined in Table 1.

na, strain not available.

nd, trait not determined.

(Kuznetsov, 1959), and it was not related to any of the other strains within the subspecies of *L. casei*. During the preparation of the Approved List of Bacterial Names in 1980, these data were neglected and Collins and co-workers (1989), on the basis of DNA/DNA hybridization data proposed to create the species *L. paracasei* for strains unrelated to *L. casei* ATCC 393<sup>T</sup>. However strain ATCC 334 was not included in the reclassification and it

maintained the name *Lactobacillus casei* even if more related to *L. paracasei* than to ATCC 393<sup>T</sup>. A first request for ATCC 334 to be designated as the neotype strain of *L. casei* in place of ATCC 393<sup>T</sup> and the rejection of the name *L. paracasei*, based on numerical analysis of total soluble cell protein and DNA–DNA hybridization experiments (Dellaglio *et al.*, 1991), was denied (Wayne, 1994). After that pronouncement, however, a number of studies were

published in which either ATCC 334 or ATCC 393 were indicated as the reference strain or the type strains for the species *L. casei*. Moreover, considering new isolates, identification procedures could result in the attribution of the name *L. casei* or *L. paracasei* depending on the reference strain used, *L. casei* ATCC 334 or *L. paracasei* subsp. *paracasei* NCDO 151<sup>T</sup>, respectively. However, these two strains are very similar, in certain analyses almost identical, although different from *L. casei* ATCC 393<sup>T</sup>. The status of valid species for *Lactobacillus zeae*, formerly '*Lactobacterium zeae*' (Kuznetsov, 1959) was accepted (Dicks *et al.*, 1996), but the epithet did not include strain ATCC 393<sup>T</sup>. A number of studies (Mori *et al.*, 1997; Zhong *et al.*, 1998; Tynkynen *et al.*, 1999; Chen *et al.*, 2000; Felis *et al.*, 2001) supported the revision of the nomenclature of the *L. casei* species group, i.e. the reclassification of strain ATCC 393<sup>T</sup> as *L. zeae* and the rejection of the name *L. paracasei*. A detailed review of the data cited here and the formal request for the change in the nomenclature of the *L. casei* species group are reported in Dellaglio *et al.* (2002). However, at present, correct taxonomic procedure would imply the attribution of names on the basis of comparison with type strains, therefore strains could be named *L. casei* if they resemble more ATCC 393<sup>T</sup> than NCDO 151<sup>T</sup>, and the relationships with strain ATCC 334 has no formal meaning.

The application of molecular techniques to the analysis of a larger collection of strains have revealed complex relationships between different strains (Vazquez *et al.*, 2005) and common misidentification of commercial strains belonging to the *L. casei* group (Deasi *et al.*, 2006).

Thus, it is evident that even for well established species, all the aspects of taxonomic analysis are not a simple and straightforward task.

#### First insights from genomic data

Very recently, the results of a program aimed at sequencing of non pathogenic LAB have been presented (Makarova *et al.*, 2006; Makarova and Koonin, 2007). Comparative analysis of genome sequences of 18 strains, representing 14 species of the order *Lactobacillales* (*L. acidophilus*, *L. brevis*, *L. casei*, *L. delbrueckii* subsp. *bulgaricus*, *L. gasserii*, *L. johnsonii*, *L. plantarum*, *L. sakei* subsp. *sakei*, *L. salivarius*, *Pediococcus pentosaceus*, *Oenococcus oeni*, *Leuconostoc mesenteroides*, *Lactococcus lactis*, and *Streptococcus thermophilus*), has revealed extensive occurrence of gene loss, duplication, and acquisition. Most notably, from a taxonomic standpoint, is the phylogenetic reconstruction, which is possible with protein sequences, instead of 16S gene sequences. This analysis, based on the concatenated alignments of four subunits of the DNA-dependent RNA polymerase and on ribosomal proteins, confirmed that *L. gasserii*, *L. johnsonii*, *L. acidophilus*, and *L. delbrueckii* belong to the same phylogenetic group while other lactobacilli are less related. These findings were in agreement with another study, based on fewer genome data (Canchaya *et al.*, 2006).

Surprisingly, the analysis of Makarova *et al.* and Makarova and Koonin revealed that *Leuconostoc* and *Oenococcus*, of the family *Leuconostocaceae*, seem to be more evolutionary related to *L. brevis*, *L. plantarum*,

*L. salivarius* and *P. pentosaceus* than *L. casei* and *L. delbrueckii* group (Makarova *et al.*, 2006; Makarova and Koonin 2007). Thus, genomic data confirm the intermixture of *Lactobacillus* and *Pediococcus*, but also open a new perspective in the evolution. Therefore these results will influence the taxonomy of the entire order *Lactobacillales* rather than only the family *Lactobacillaceae*, as the depicted evolutionary scenario contradicts the scheme obtained by 16S rRNA gene sequence analysis. However, it must be noted that evolutionary conclusions are drawn on the basis of genome data for 9 (Makarova *et al.*, 2006) and 12 strains (Makarova and Koonin, 2007), while the two families (*Lactobacillaceae* and *Leuconostocaceae*) group 144 species. Therefore, the addition of other genome sequences to the dataset could change the proposed scheme, even if the discrepancy between the genome-derived and the ribosomal-based schemes is significant on this small dataset (data not shown).

#### The genus *Bifidobacterium*

Bifidobacteria are Gram-positive polymorphic branched rods that occur singly, in chains or clumps. They are non-spore-forming, non-motile, and non-filamentous. They are anaerobic and chemoorganotrophs, having a fermentative type of metabolism. They produce acid but not gas from a variety of carbohydrates. They are catalase negative, with some exceptions (*Bifidobacterium indicum* and *Bifidobacterium asteroides* when grown in presence of air). Their genome GC content varies from 42 to 67 mol% (Biavati and Mattarelli, 2001).

They occur in animal and human habitats, in particular they have been isolated from feces, rumen of cattle, sewage, human vagina, dental caries and honey bee intestine.

According to the *Taxonomic Outline of the Prokaryotes* (Garrity *et al.*, 2004), the genus *Bifidobacterium* belongs to the phylum *Actinobacteria*, class *Actinobacteria*, subclass *Actinobacteridae*, order *Bifidobacteriales*, family *Bifidobacteriaceae*. The other genera belonging to this family are: *Aeriscardovia*, *Falcivibrio* (see below), *Gardnerella*, *Parascardovia* and *Scardovia*.

To date, species included in the genus *Bifidobacterium* are 29: *Bifidobacterium adolescentis*, *Bifidobacterium angulatum*, *Bifidobacterium animalis* (with the two subspecies *B. animalis* subsp. *animalis* and *B. animalis* subsp. *lactis*), *Bifidobacterium asteroides*, *Bifidobacterium bifidum* (type species), *Bifidobacterium boum*, *Bifidobacterium breve*, *Bifidobacterium catenulatum*, *Bifidobacterium choerinum*, *Bifidobacterium coryneforme*, *Bifidobacterium cuniculi*, *Bifidobacterium dentium*, *Bifidobacterium gallicum*, *Bifidobacterium gallinarum*, *Bifidobacterium indicum*, *Bifidobacterium longum*, *Bifidobacterium magnum*, *Bifidobacterium merycicum*, *Bifidobacterium minimum*, *Bifidobacterium pseudocatenulatum*, *Bifidobacterium pseudolongum* (with the two subspecies *B. pseudolongum* subsp. *pseudolongum* and *B. pseudolongum* subsp. *globosum*), *Bifidobacterium psychraerophilum*, *Bifidobacterium pullorum*, *Bifidobacterium ruminantium*, *Bifidobacterium saeculare*, *Bifidobacterium scardovii*, *Bifidobacterium subtile*, *Bifidobacterium thermacidophilum* (with the two subspecies *B. thermacidophilum* subsp.

*thermacidophilum* and *B. thermacidophilum* subsp. *porcinum*) and *Bifidobacterium thermophilum*. The other genera group very few species: *Aeriscardovia aeriphila* in *Aeriscardovia*, *Gardnerella vaginalis* in *Gardnerella*, *Parascardovia denticolens* in *Parascardovia* (former *Bifidobacterium denticolens*) and *Scardovia inopinata* (former *Bifidobacterium inopinatum*) in *Scardovia*. Species belonging to the genus *Falcivibrio*, i.e. *Falcivibrio grandis* and *Falcivibrio vaginalis* have been transferred to the genus *Mobiluncus*, to the species *Mobiluncus mulieris* and *Mobiluncus curtisii*, respectively (Hoyles *et al.*, 2004), which belongs to the order *Actinomycetales*, suborder *Actinomycineae*, family *Actinomycetaceae* (Garrity *et al.*, 2004).

The phylogenetic relationships among the genera of the family *Bifidobacteriaceae* and the genus *Mobiluncus* are depicted in Fig. 2, on the basis of 16S rRNA gene sequence analysis. For a long time bifidobacteria have been considered relatives to lactobacilli, and the correlation is supported by the analysis of the murein structure (Kandler and Lauer, 1974). However, Poupard *et al.* (1973) and other researchers were the first to postulate that they are more related to *Actinomyces*, which has been confirmed by analysis of 16S rRNA sequences (Woese, 1987).

Bifidobacteria degrade hexoses through a peculiar metabolic pathway, the so-called *bifid shunt*, i.e. the fructose-6-phosphate pathway, the key enzyme of which is fructose-6-phosphoketolase (EC 4.1.2.2). This enzyme was considered a taxonomic character for the identification on the genus level (Biavati and Mattarelli, 2001), but, due to the reclassification of *Bifidobacterium* species into new genera, it can be considered a taxonomic marker for the Family *Bifidobacteriaceae*. A complete survey of identification methods for bifidobacteria has been compiled by Ventura *et al.* (2004) and it is still valuable as very few changes have occurred in this group of bacteria in the last years.

With regard to different phylogenetic analyses, i.e. analyses obtained with different methods and models, we can affirm that there are several species associated in groups, within the genus *Bifidobacterium*. Groups have been named considering the oldest name included in the group; they are: ***B. adolescentis* group** (which includes *B. adolescentis*, *B. angulatum*, *B. catenulatum*, *B. dentium*, *B. merycicum*, *B. pseudocatenulatum* and *B. ruminantium*), ***B. pullorum* group** (*B. gallinarum*, *B. pullorum* and *B. saeculare*), ***B. asteroides* group** (*B. asteroides*, *B. coryneforme*, *B. indicum*), ***B. boum* group** (*B. boum*, *B. thermacidophilum*, *B. thermophilum*), ***B. pseudolongum* group** (*B. animalis*, *B. choerinum*, *B. cuniculi*, *B. gallicum*, *B. pseudolongum*). Species *B. breve* and *B. longum* form a couple, as well as *B. minimum* and *B. psychroaerophilum* (less supported). *Bifidobacterium bifidum*, *B. magnum*, *B. scardovii* and *B. subtile* form distinct branches. A similar classification scheme has been obtained with phylogenetic analysis based on partial *hsp60* gene sequences (Jian *et al.*, 2001). Recently, a phylogenetic re-analysis performed with a multilocus sequencing approach has been performed (Ventura *et al.*, 2006): the phylogenetic structure of the genus has

been almost confirmed, even if an improvement in the discrimination of related species has been achieved.

Bifidobacterial strains exhibiting probiotic properties belong to the species *B. adolescentis*, *B. animalis*, *B. bifidum*, *B. breve*, '*B. infantis*' and *B. longum*: it can be noted that those species are not phylogenetically related, thus underlining the strain-specificity of those characteristics.

Taxonomic issues in the genus *Bifidobacterium* concern the species *B. longum*-*B. infantis*, recently solved with union under the name *B. longum* and the recognition of three biotypes, the *infantis* type, the *longum* type and the *suus* type, by molecular methods (Sakata *et al.*, 2002). However Young *et al.* (2004) recently found interesting results about the interaction of *B. infantis* and *B. longum* with dendritic cells: they found that the ATCC/DSMZ cultures of these two species have different interactions with those cells and then they should be kept distinct.

Another debated issue is the relationship between *B. animalis* and *B. lactis*, the latter recently reclassified as *B. animalis* subsp. *lactis* (Masco *et al.*, 2004).

Finally, the differentiation of *B. indicum*, *B. coryneforme* and *B. asteroides* have been raised: *B. indicum* and *B. asteroides* could be clearly distinguished, but *B. indicum* and *B. coryneforme* cannot (E. Lauer, personal communication). A paper of Temmerman *et al.* (2003) confirmed, by DGGE analysis, the high relatedness of the three species, but the multilocus sequence analysis of Ventura *et al.* (2006) was successful in their distinction, even if their close relatedness was confirmed.

### From species to strain

Correct identification at species level could be an indication of the suitability of bacterial strains for industrial application, however performances in an applied context are often strain-dependent. Therefore, it is of primary importance to use techniques able to reveal strain peculiarities. Once again, the most powerful methods are molecular ones and a number of techniques have been developed and applied to typing of strains belonging to LAB. Methods could be DNA-based or phenotypic: DNA based techniques could be PCR-based such as RAPD-PCR, AFLP, etc., or could exploit properties of the organism itself, e.g. the number of endonuclease recognition sites in the genomes (Pulsed-Field Gel Electrophoresis, PFGE) or similarity of DNA primary structure (DNA-DNA hybridization, Southern hybridization with different probes). Phenotypic molecular methods include techniques such as SDS-PAGE of whole cell proteins. Probably the most applied techniques are the PCR-based methodologies, which could target the genome randomly or pseudo-randomly (RAPD-PCR), or could target specific sites in the bacterial genome (ERIC-PCR, Enterobacterial Repetitive Intergenic Consensus-based polymerase chain reaction, or REP-PCR, Repetitive Extragenic Palindromic sequence-based polymerase chain reaction). Results of different techniques could be combined and evidences for the presence of genome similarity clusters in a species may be observed.

### Other functionally related species of LAB

Two species, belonging to lactic acid bacteria, play an outstanding role in the production of fermented milks

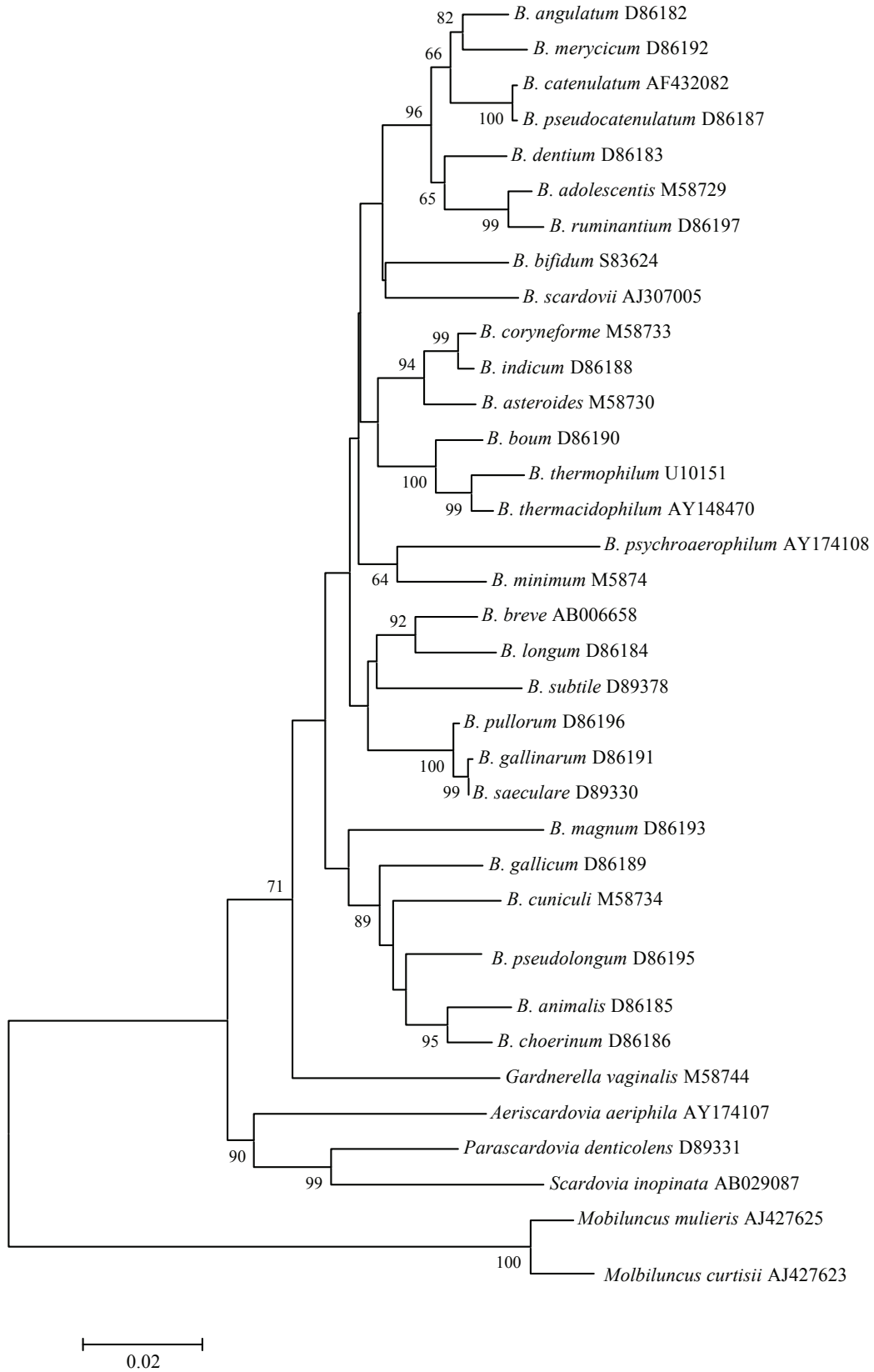


Fig. 2 Phylogenetic tree based on 16S rRNA gene sequence analysis showing the phylogenetic relationships among species of the Family Bifidobacteriaceae. Bar indicates number of nucleotide substitution per site. See Fig. 1 for details of procedure of tree inference.

and dairy products: *Streptococcus thermophilus* and *Lactococcus lactis*. Even if they are often not considered as probiotics, they are extraordinary actors in industrial dairy fermentations, for the obtainment of products used as vehicle of probiotic strains. In this review of the taxonomy of bacteria interesting for their positive role, a few information will be given on the taxonomic placement and molecular identification methods usually applied for the identification of the aforementioned two species.

The genera *Lactococcus* and *Streptococcus* form the Family *Streptococcaceae* of the Order *Lactobacillales* in the Class *Bacilli* of the Phylum *Firmicutes*.

The genus *Streptococcus* contains about 60 species and a number of them is known for their pathogenicity. The species *S. thermophilus*, however, is constituted by GRAS microorganisms, very essential in the dairy manufacturing which makes it one of the most commercially important of all LAB. It is Gram-positive, catalase negative, non-motile, non-spore-forming and homofermentative. In association with *L. delbrueckii* subsp. *bulgaricus*, it is used as starter culture for the production of yogurts. The role of this product in well-being and therefore the attribution of beneficial properties (alleviation of symptoms of lactose intolerance and other gastrointestinal disorders) to those two bacteria trace back to the pioneer of probiotics, Ilya Ilyich Metchnikoff, in the book *The Prolongation of Life* (1908): he wrote that consumption of live bacteria, such as '*L. bulgaricus*', now *L. delbrueckii* subsp. *bulgaricus*, and *S. thermophilus*, in the form of yogurt was beneficial for general health, besides gastrointestinal health, and for longevity. At present, besides yogurt production, *S. thermophilus* is used in formulations together with bifidobacteria and other LAB (e.g. *L. casei*, *L. johnsonii*, *L. plantarum*, *L. rhamnosus*, *L. reuteri*) with positive results, in such cases it is almost impossible to distinguish which of the two different bacterial strains have had the most relevant effect (e.g. Saavendra *et al.*, 1994).

This species have twice undergone a revision of nomenclature in recent years: *S. thermophilus* (Orla-Jensen, 1919) had been reclassified as *S. salivarius* subsp. *thermophilus* by Farrow and Collins (1984) but the definition of its status of separate species have definitively been established by Schleifer *et al.* (1991) with the name of *Streptococcus thermophilus*, *nomen revictum*. Therefore formal designation of that species is *Streptococcus thermophilus* (Orla-Jensen 1919) Schleifer *et al.* 1995, comb. nov., the year referring to the date of validation list number 54 on International Journal Systematic Bacteriology. Its detection in dairy products could be achieved through a specific amplification of *lacZ* gene, a rapid and reliable PCR-based technique (Lick *et al.*, 1996).

Genome sequences of three *S. thermophilus* strains have recently become available (Bolotin *et al.*, 2004; Makarova *et al.*, 2006) and have permitted insights in the evolutionary trends of the species also in relation to pathogenic species of the same genus (Hols *et al.*, 2005).

The genus *Lactococcus* groups bacteria formerly referred to as mesophilic lactic streptococci. It is constituted by five species and the most important for dairy fermentation is *L. lactis*, which is divided into

three subspecies, namely *L. lactis* subsp. *cremoris*, *L. lactis* subsp. *hordniae* and *L. lactis* subsp. *lactis*, which include the biovar *diacetylactis*. One of the most reliable methods to detect and identify the *L. lactis* species and its subspecies has been proposed in 1997 (Beimfohr *et al.*, 1997): it is based on specific amplification of different regions of the histidine biosynthesis operon and it also allows the discrimination of the biovar *diacetylactis*.

Considering the taxonomy of the species, an open issue is the taxonomy of the species *L. lactis*, and in particular of the subspecies *lactis* and *cremoris*. In fact, two genomic population are clearly distinguishable, which do not match with the infra specific subdivision obtained considering phenotypic traits (van Hylckama Vlieg *et al.*, 2006, and references therein).

*Lactococcus lactis* is probably the most economically important LAB species and it is also a very important model organism for low GC Gram-positive bacteria. This is still stimulating extensive research efforts which have resulted in the genome sequencing of three strains of the species (Bolotin *et al.*, 2001; Makarova *et al.*, 2006; Wegmann *et al.*, 2007). Hopefully, comparative genome sequence analysis will clarify also the relationships within the species, which can be exploited for the establishment of a more predictive taxonomic scheme.

## Concluding remarks

### *Resources for taxonomists and users*

It has been explained how difficult a taxonomic analysis could be. Taxonomy have often been seen as a boring and, in some way, useless discipline, but it is now being recognized as very important also in an applied context: it creates the common language in scientific communication when microorganisms are involved and that is very important also in the industrial exploitation of microorganisms (e.g. labelling of products). Sometimes those who are unfamiliar with taxonomy find it very difficult to deal with names changing over time, for example new species names, or nomenclature changes of known strains. Therefore, it seems useful to briefly explain how to retrieve reliable taxonomic information for people not directly involved in taxonomy.

The body that oversees the nomenclature of prokaryotes is the International Committee on Systematics of Prokaryotes (ICSP) ([www.the-icsp.org](http://www.the-icsp.org)). It determines the rules by which prokaryotes are named. The rules are collected in the Bacteriological Code (Lapage *et al.*, 1992, see below). Taxonomic issues of particular taxa are dealt with by a number of subcommittees. The LAB are included in the scope of different Subcommittees: the Subcommittee on the taxonomy of *Bifidobacterium*, *Lactobacillus* and related organisms covers taxonomic matters concerning almost all the genera of the order *Lactobacillales*, while the Subcommittee on the Taxonomy of Staphylococci and Streptococci includes in its scope, among others, the genera *Lactococcus*, *Streptococcus* and *Enterococcus*.

The Judicial Commission is the body of the committee which issues opinions concerning taxonomic matters, revision to the Bacteriological Code, etc.

The journal which officially publishes taxonomic papers (i.e. description of novel species/new genera, renaming, request for opinions, opinions of the Judicial Commission, etc.) is the *International Journal of Systematic and Evolutionary Microbiology* (<http://ijs.sgmjournals.org/>), former *International Journal of Systematic Bacteriology*. All new names have to be published in the *IJSEM* either by being described there, or, if described elsewhere, by placing them there in the 'Validation Lists'. All bacterial names are therefore found in a single journal, even if taxonomic descriptions might be present elsewhere.

The most recent version of the Bacteriological Code has been published by Lapage *et al.* (1992): it contains Rules (obligatory) and Recommendations (just guides to good practice) on how to deal with nomenclature of bacterial taxa. There are sections on how to describe, name and publish on a novel bacterium, and on how to request the Judicial Commission to look into nomenclature problems. It also lists names that have been protected (conserved) and those that must be rejected, and advises on naming of infrasubspecific divisions. A few amendments to the Code have been made since its publications and they can be found in issues of the *IJSEM*.

Bacterial nomenclature can be checked online on the DSMZ (German collection of microorganisms and cell cultures, <http://www.dsmz.de/bactnom/bactname.htm>) or on the internet pages compiled by J.P. Euzéby and updated after each issue of *IJSEM* (<http://www.bacterio.cict.fr/>).

Considering classification, the reference book is the *Bergey's Manual of Systematic Bacteriology*, now at its second edition (2001), which provides information of genetic and phenotypic traits of all known taxa and arrange them in a hierarchical scheme on the basis of phylogenetic relatedness, when possible.

It should then be clear that nomenclature is regulated, while taxonomy is a matter of experience and agreement among scientists. The permanent link between a name and a species is constituted by the type strain, i.e. the strain chosen as a representative of the species at the time of the first description. Novel species descriptions require the deposit of the type strain in at least two recognized culture collections (e.g. ATCC, [www.atcc.org](http://www.atcc.org); LMG, [www.bespo.be/bccm](http://www.bespo.be/bccm); DSMZ, [www.dsmz.de](http://www.dsmz.de); JCM, <http://www.jcm.riken.jp/>), to make it available to the scientific community for study. This requirements may sometimes be in conflict with patent requirements but it is essential, as culture collections are the only international organizations devoted to storage and preservation of biodiversity.

Moreover, type strains have to be included in all the comparative studies among different species, to guarantee the significance of the study and the proper use of names.

#### *The impact of culture-independent techniques in the discovery of unknown biodiversity*

It is well known, among microbiologists, that the great majority of prokaryote biodiversity is uncultured and therefore unknown (Rosselló-Mora and Amann, 2001; Siezen *et al.*, 2004). The investigation of the uncultured has increased in the last years, as biodiversity is thought

to include species and strains which could be source of innovation in biotechnology (Bull *et al.*, 1992). The most applied techniques for this purpose are culture-independent techniques such as DGGE (Denaturing Gradient Gel Electrophoresis) and TGGE (Temperature Gradient Gel Electrophoresis) (Muyzer and Smalla, 1998), based on PCR amplification of partial region of 16S rRNA genes or other targets directly from environment. Even if PCR-based methods have some drawbacks (e.g. Wintzingerode *et al.*, 1997), such analyses have often brought important advancements in the comprehension of biodiversity and ecology of investigated niches. Very recently another technique, the T-RFLP (Terminal-restriction fragment length polymorphism), has been successfully applied to analyse the fecal microbiota of children with different lifestyles and have revealed the presence of a dominant but as-yet-uncultured LAB species (Dicksved *et al.*, 2007).

Considering probiotic bacteria, the unknown majority may include bacteria which may exert probiotic actions: culture-independent techniques may help in their identification and optimization of growth media for their isolation. This is necessarily the first step to obtain pure cultures which could be studied and eventually used as probiotics. Moreover, the uncultured microflora may include unknown and/or undesirable bacteria: in this case culture-independent techniques may produce kind of 'intestinal fingerprints' which give an idea of the effect on the intestinal microflora obtained with the administration of probiotics in clinical trials.

Those methods are based on sequence data and allow identification of the biodiversity of the investigated environment through sequence comparisons in public databases. Such methods are useful for the optimization of culture conditions which could allow the isolation of taxa not previously isolated. If the strain bearing a very peculiar sequence could not be isolated in pure culture, it cannot be described as a new species but it can be indicated with the *Candidatus* state, according to *Rules of the Bacteriological Code* (Lapage *et al.*, 1992).

#### *The role of genome sequence data in bacterial taxonomy*

Taxonomy is an evolving process, which tries to group biodiversity in an artificial scheme. Phenomena such as horizontal gene transfer and adaptation to ecological niches may influence our understanding of biodiversity and therefore its classification, leading to erroneous results that can be refuted when additional information are retrieved for the organisms under study. Intuitively, the ultimate frontier of that 'additional information' is the complete genome sequence analysis of more than one strain for each species.

Comparison of genome sequences of bacteria is expected to give indication of their evolutionary dynamics and finally allow a really 'natural' classification scheme. Genome sequences available for lactobacilli and bifidobacteria are increasing and revealing important insights. The analyses of differences in gene content, gene order on the chromosome (sinteny), a phylogenetic analysis with a 'supertree' approach (i.e. based on virtually all available genes) and other analytic approaches concerning nucleotide composition, codon

usage etc. could give information at different taxonomic levels. Sequencing of any strain will probably remain an unfeasible task, but the discovery of trends of evolution on the basis of a sample of organisms will probably improve our understanding of microbial biodiversity: recent observations suggest that LAB have undergone considerable gene loss and acquisition, which can be reconstructed. Moreover, comparative analysis of related strains have revealed that adaptation to different niches, milk in particular, has lead to an extensive reductive evolution and gene decay, evident in *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* (Hols *et al.*, 2005; van de Guchte *et al.*, 2006).

The genomic era is therefore very promising also for taxonomy, but two points must be carefully evaluated: (i) the number of species described is much larger than genome sequences available or interesting to be obtained, thus the risk is to draw conclusions biased by the low number of analysed sequences; (ii) the risk of establishing a parallel standard, where species names and properties are associated to strains which genome has been sequenced, regardless of the type strains. Strains chosen for genome sequencing are usually laboratory model strains or commercially interesting organisms, but only the type strains are conventionally chosen to represent the species; the establishment of a parallel standard could potentially generate considerable confusion in the scientific community (Felis *et al.*, 2007).

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